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THE MAKING OF A PHAGE

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Up from the sewer

As a consequence of the structural work on DNA, the concerted investigations on the T even phages and the in vitro work on DNA polymerase, the replication of DNA-containing viruses was understood in outline by the end of the fifties. However, at this time virtually nothing was known about the propagation of RNA viruses, mainly because biochemical work on plant and animal viruses entailed considerable technical difficulties.

The discovery of the RNA-containing coliphage f2 in Manhattan sewage by Loeb and Zinder in 1960 [1] presaged rapid advances in the field and aroused great interest among virologists. Excursions were undertaken to sewers of cities throughout the world, bringing to light numerous further isolates of RNA phages – f_{can} in Canberra, R17 in Philadelphia, M12 in Munich, Qβ in Kyoto, MS2 in Berkeley, to mention but a few. These phages proved to be ideal objects for biochemical investigations. Not only do they multiply rapidly, providing impatient experimenters with fast (if not always clear) results, but they also subvert almost half of their host's metabolism to their own needs, which greatly aids the biochemical analysis of the phage-specific processes. Last but not least, RNA phages can be grown and purified by the tens of grams, providing adequate amounts of material for structural and functional analyses.

All phages isolated in the USA and in Europe are closely related, belonging to serological group I. They show only slight immunological differences, which can be related to a few amino acid substitutions in the coat protein. The Japanese phages Qβ and VK belong to the serological group III, which shows no cross-reactivity with group I [2–4]. The structure of the coat protein of Qβ differs widely from that of group I phages, however a limited homology may be detected between the two groups, both in the amino acid sequences of the coat proteins [5–7] and in the nucleotide sequences of the RNAs [8–10]. Phages of group II, IV and V have not been investigated extensively.

Some vital statistics

RNA phage particles are roughly spherical with a diameter of about 25 nm [11], and are thus somewhat larger than *E. coli* ribosomes. The single-stranded RNA genome consists of 3500 (group I) to 4500 (group III) nucleotides [12,13] and has a notably compact, probably unique secondary and tertiary structure. It is surrounded by about 180 coat protein molecules; in addition, each particle contains one molecule of maturation (A or, in Qβ, A_2) protein, which is essential for the binding of the phage to its host [14–16]. Phage Qβ contains a few molecules of a second virus-specific protein (called IIa or A_1 [17]) which is essential for its infectivity (Hofstetter, Monstein and Weissmann, unpublished), for as yet unknown reasons.

Classical genetic analysis of group I and group III phages, involving the isolation of conditionally lethal mutants and their classification by complementation analysis identified three cistrons, one coding for maturation protein, a second for coat protein and the third for the β subunit of the viral RNA polymerase [18–20]. Since recombination among RNA phages has never been detected, the order of the cistrons had to be determined by nucleotide sequence analysis and other chemical and biochemical approaches [21–26].

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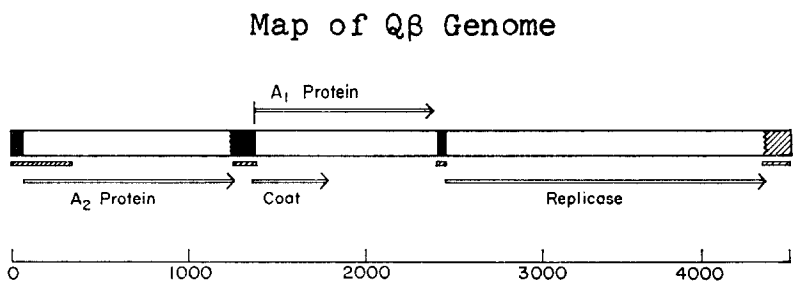

Fig. 1. Map of the the Qβ genome. Non-translated regions are dark; the locations of the cistrons are marked by arrows. The areas of known nucleotide sequence are indicated by the narrow bars under the map. Based on refs. [24–26].

The map of Qβ RNA is shown in fig. 1. Two remarkable features deserve comment. The first regards the subdivision of the genome into translatable and non-translatable sections. At both ends of the RNA there exist stretches of at least 60 nucleotides, and between any two cistrons segments of 20 or more nucleotides, none of which are translated into protein [9, 26–28]. The genome of group I phages has a similar structure [21–22]. While the regions immediately preceding the cistrons are required for the initiation and regulation of protein synthesis, the function of the longer untranslatable segments at the ends of the genome is not known. It is however believed that these regions are essential for phage viability, because as judged by comparing RNA sequences of different group I phages, their primary structure is more strictly conserved than that of the cistrons [26,29]. The subdivision of the genome into translatable and silent regions is not peculiar to the RNA phages, where it was first discovered, but applies also to bacterial and eukaryotic genomes. The other remarkable feature, so far observed only in phage Qβ, is the utilization of one cistron for the synthesis of two distinct proteins (coat and A_1 protein) as described in more detail below.

Nucleotide sequence analysis of Qβ RNA and the RNA of group I phages (mainly MS2) is under way. In the case of Qβ RNA, about 20% of the sequences have been established. More than 30% of MS2 RNA, including all of the coat protein cistron has been elucidated (for detailed reviews, see [26, 30]).

Launching the invasion

RNA phages will infect only so-called male (or F^+) strains of *E. coli* [1]. These bacteria carry a characteristic appendage, the F^+ pilus, to which the phages can attach in large numbers [31]. Under unfavorable culture conditions the pilus is not grown and the bacteria enjoy temporary resistance against their venereal disease. After the attachment step the RNA is released from the coat protein shell [32] by an as yet unknown mechanism and penetrates into the bacterium carrying the maturation protein with it [33,34]. It has been proposed that the RNA strand travels into the host through the bore of the pilus [35], but there is no conclusive evidence to support this claim. Another proposal would have the pilus retract into the bacterium, pulling the attached phage particle to the bacterial surface, where the RNA would enter the cell through a pore in the cell wall [36]. It is interesting to note that purified, protein-free RNA can be introduced into any strain of *E. coli*, both F^+ or F^-, if the cell wall is first damaged with lysozyme; the infection elicited by this artificial procedure results in the synthesis of complete, normal phage particles [37,38]. It is thus clear that none of the phage particle proteins are required for the replication of the virus.

The take-over

The first stage in the take-over of the host's synthetic machinery consists in the attachment of the viral RNA to the host's ribosomes [39]. The resulting polysome produces virus-specific proteins, among others the β subunit of the viral RNA polymerase. Once this enzyme has been formed, it uses the viral RNA as template for the synthesis of more phage RNA. Part of this progeny RNA is used as messenger for further virus-specific protein synthesis, while part of

it associates with the viral capsid proteins to form progeny particles. Although the phage is not provided with specific mechanisms for shutting off host-specific synthesis, the formation of host-specific macromolecules is partially inhibited as a consequence of phage infection [40]. The extent of this inhibition varies, depending on the strain of the infecting phage, and is probably the result of a competition between phage and host-directed syntheses for ribosomes, substrates and other essential components.

Custom-made proteins

It is remarkable that the different viral proteins are synthesized in very different molar amounts despite the fact that all cistrons are represented once on one RNA strand. Thus, in the case of phage MS2 the ratio of synthesis of coat protein to A protein to replicase β subunit is about 20:2:1 [41,42] reflecting to some extent the phage's requirements for these proteins. How does this regulation come about? Each cistron has its own ribosome binding site, at which initiation of translation may be controlled [43–47], as well as its own termination site [21,29,49]. We may classify the regulatory processes into three categories, namely modulation of initiation, translational repression and modulation of termination.

i) *Modulation of initiation.* Ribosome binding occurs with quite different efficiencies at the three binding sites of the viral RNA [43]. This is due to differences in the nucleotide sequences of the binding sites as well as in the secondary and possibly tertiary structure at and around these regions. Beside the initiation triplet no characteristic sequence or secondary structure is common to all ribosome binding sites (cf. review [26]).

Ribosome binding and initiation are also subject to control by the host's protein initiation factor system (IF3 and the interference factors) (cf. review [50] and [51–56]). Both in vitro and in vivo ribosome binding and chain initiation initially occur predominantly at the coat cistron, while initiation at the replicase cistron takes place only while the coat cistron is being translated [18,43,57]. This is probably due to an unfolding of the RNA by the translating ribosomes which makes the binding site at the replicase cistron

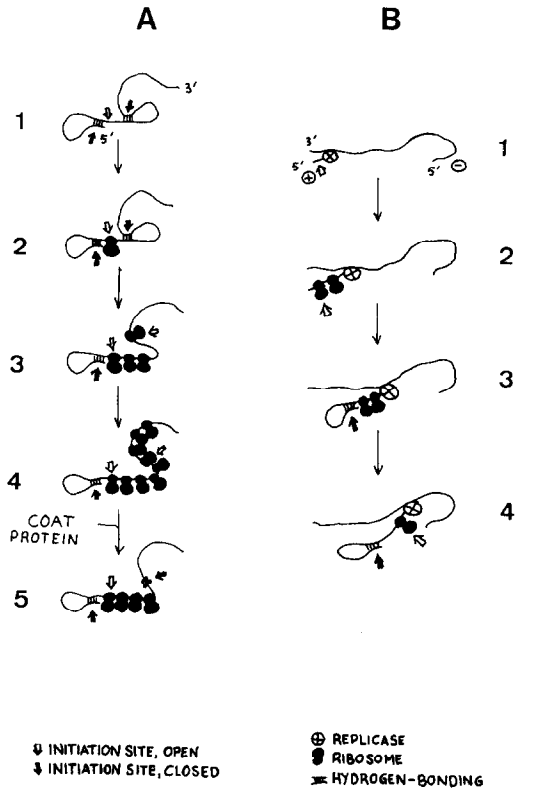

Fig. 2. Translation on mature and nascent phage RNA. (A) Translation on mature RNA (1). Only the coat initiation site is accessible to ribosomes (2). As the coat cistron is translated, ribosomes can attach at the replicase cistron (3) giving rise to a polysome on which the coat and replicase, but not the maturation cistron are translated (4). During later stages of the infective cycle coat protein accumulates in the cell and binds to the RNA so as to block protein initiation at the replicase cistron (5). (B) Translation on nascent RNA. The viral replicase initiates synthesis of a plus strand at the 3′ end of a minus strand (1). When the ribosome binding site of the maturation (or A) protein has been formed, ribosomes attach and begin translation of this cistron (2). As plus strand synthesis progresses, the plus strand assumes a secondary structure which prevents access of ribosomes to the A cistron (3). At this point initiation of protein synthesis is now possible only at the coat cistron (4), as in the case of mature RNA (A). (See text for references).

– which is normally hydrogen-bonded to the beginning part of the coat cistron [29] – available for initiation (fig. 2A). No initiation of maturation (A_2) protein at all is observed on mature (i.e., complete,

single-stranded) phage RNA [44]. It is believed that the ribosome binding site of this cistron, which is located close to the 5′ end of the RNA, is available to ribosomes only as long as the RNA is at an early stage of synthesis, and becomes unavailable when the nascent strand is further elongated and folded into its mature secondary and tertiary structure (fig. 2B) [47,58,59]. Thus the A_2 cistron can be translated for only a fraction of the viral RNA's intracellular lifetime, limiting the amount of A_2 protein that can be synthesized.

ii) *Translational repression.* The mechanism described above would lead to a coordinated synthesis of the viral proteins in a defined ratio throughout the replication cycle. In reality, synthesis of replicase is almost completely turned off about midway through the cycle [41]. This shut-down is due to the accumulation of coat protein, which binds at the intercistronic region preceding the replicase cistron and prevents further initiation of protein synthesis at this point (fig. 2A) [61–63]. A further mode of translational repression plays an important role in the early stages of infection. As mentioned above, phage RNA serves first as a messenger for protein synthesis and subsequently as template for its own replication. During the first process the viral RNA is present as a polysome, with ribosomes travelling in the 5′ to 3′ direction. In replication, the viral polymerase advances along the template in 3′ to 5′ direction, i.e. on a collision course with translating ribosomes. Since the polymerase cannot dislodge ribosomes bound to the viral RNA this sequence of events would lead to disaster, at least as far as the virus is concerned. How is this difficulty overcome? As shown in fig. 3, the viral polymerase has a strong affinity for the intercistronic region preceding the coat cistron and on binding at this position prevents the attachment of ribosomes [59,64a]. As a consequence further initiation of translation cannot take place at either the coat or the replicase cistron while ribosomes already engaged in translation can terminate synthesis and detach [64]. Since the A_2 cistron is not translated on mature RNA anyway this means that the viral RNA is then cleared of ribosomes and becomes available as template for its own replication.

iii) *Modulation of termination.* As mentioned above, the middle cistron of Qβ RNA codes for both the coat protein and A_1 protein. Coat protein is formed when translation terminates at a UGA triplet about 400 nucleotides from the initiation site; if termination is suppressed at this position, translation continues for about another 800 nucleotides [65–68] and is terminated at a double stop signal UAGUAA (Billeter, Weber and Weissmann, unpublished results). Since even wild. type *E. coli* always contains a low level of suppressor tRNA which inserts an amino acid, probably tryptophan, at the UGA triplet [69], about 3% of the translating ribosomes run to the end of the cistron producing 3 molecules of A_1 for every hundred molecules of coat protein. Thus, phage Qβ has evolved to take advantage of a peculiarity of the host's translational machinery to regulate the relative amounts of coat and A_1 protein produced.

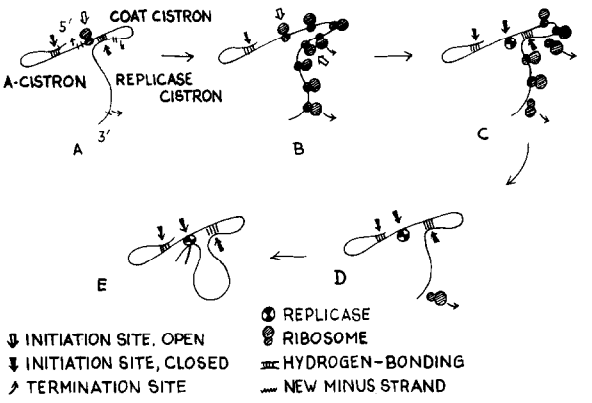

Fig. 3. Transition of phage RNA from polysome to replicating complex – repressor function of Qβ viral replicase. (A) Ribosomes attach to the RNA at the coat initiation site. The initiation site of the replicase cistron is unavailable because of the secondary structure of the RNA. (B) Translation of the coat cistron ensues and the initiation site of the replicase cistron is exposed. The replicase cistron is translated. (C) When replicase becomes available, it attaches to the initiation site of the coat protein and blocks attachment of ribosomes in this position. The RNA refolds, preventing initiation at the replicase cistron. (D) The RNA is cleared of ribosomes. (E) Replicase can now attach to the 3′ terminus and initiate synthesis of the minus strand. The A cistron initiation site is at all times unaccessible to ribosomes because of the secondary structure of the mature RNA (cf. fig. 2) (from ref. [64]).

The mechanism of RNA replication is almost as expected

Several different mechanisms of viral RNA replication have been considered at one time or another. In

the early sixties a direct copying mechanism was in vogue, in which the template would direct the viral polymerase to insert the identical, rather than the complementary nucleotide, thereby circumventing the Watson–Crick principle of nucleic acid replication [70]. While there is no strong theoretical reason to exclude such a mechanism a priori, no experimental support whatsoever was found for this proposal. Another mechanism envisaged in the early stages of the investigation was one whereby the viral RNA would be transcribed from the host's DNA. Although this esoteric pathway was soon excluded for RNA phages [71], it was, a decade later, proven to apply in the case of the RNA tumor viruses [72]. Of course, the most popular mechanism at all times was one involving the synthesis of a minus strand, i.e., a strand complementary to the viral RNA, to form a double-stranded 'replicative form' which would subsequently serve as template for the synthesis of progeny phage RNA, in analogy to the sequence of events found for the single-stranded DNA phage ϕX 174. This model was in fact so appealing that it has kept its place even in recent textbooks, despite the fact that it is inaccurate in several non-trivial respects. While indeed the first step of RNA replication is the synthesis of a minus strand [73,74], this RNA is synthesized in a single-stranded configuration and does not form a double helix with the plus strand [74–80]. After its completion the single-stranded minus strand is used as template for the formation of progeny phage RNA, which is also synthesized and released in a single-stranded form (fig. 4) [74]. Although nucleotide selection most probably involves Watson–Crick hydrogen-bonding between the bases of the template and the incoming substrates (proof for this is not yet forthcoming!), these interactions must be undone shortly after the base is incorporated; the mechanism responsible for this is not understood. The replicating complex, consisting of enzyme, single-stranded template and single-stranded product is a labile structure, inasmuch as it is easily converted into a double-stranded RNA (which is completely inactive as template for RNA synthesis) [74,76]. The lability of the replicating complex accounts for the fact that considerable amounts of biologically inactive double-stranded RNA accumulate in infected cells late in the infectious cycle [81,82]. Synthesis of both the plus and the minus strands is carried out by one and the same RNA-dependent RNA polymerase, or replicase [60,74,83], however an additional host-specific fac-

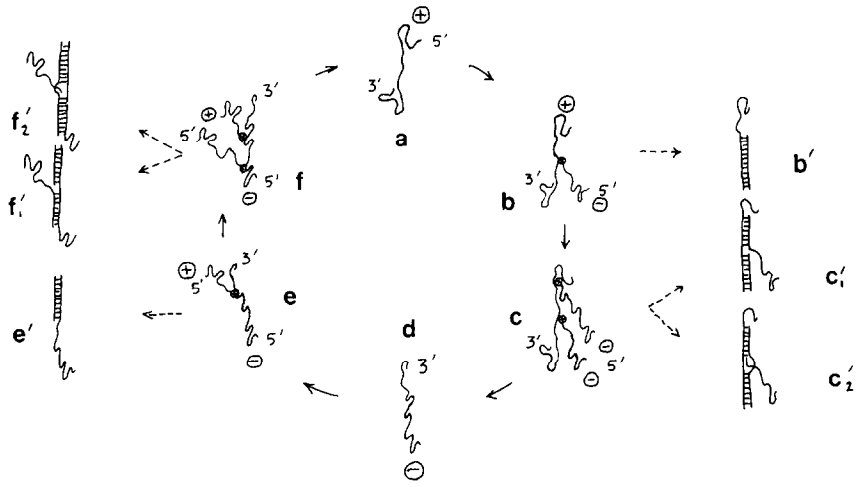

Fig. 4. Replication of phage RNA. In the first step of RNA replication the plus strand (a) is used as template for the synthesis of minus strands. The replicating complexes (b and c) consist of a single-stranded template, one or more single-stranded nascent minus strands and Qβ replicase molecules. The resulting product, a single-stranded minus strand (d) is then used as template for the second step of RNA synthesis. The intermediates, the replicating complexes (e) and (f) have a structure similar to that of the complexes of the first reaction step and yield single-stranded plus strands (a) as product. The intermediates (b), (c), (e) and (f) are metastable and collapse spontaneously or under the influence of external agents, to yield the partially double-stranded structures (b'), (c'), (e') and (f'). Modified from [74].

tor, HFI, is required to initiate synthesis of the minus strand on the plus strand template [60,84]. The amount of plus strands synthesized in vivo exceeds that of minus strands about 10-fold [82]. The most likely reason for this asymmetry may be that ribosomes and, particularly in the later part of the infectious cycle, coat protein molecules attach to plus strands, making them less available as templates.

The viral replicase, which has a molecular weight of about 215 000, consist of four different subunits, only one of which, the β subunit, is coded for by the viral genome [85–87]. The other three are host-specific polypeptides, all of which, interestingly enough, are normally involved in some aspect of protein and not of RNA synthesis. Replicase subunits γ and δ are identical with the elongation factors Tu and Ts respectively [88], while subunit α is factor *i* [89], which plays a role in the specificity of the polypeptide initiation process [53,54]. The precise function of each subunit in RNA synthesis is not known, however α is required for efficient binding of the enzyme to the plus, but not to the minus strand [90], and it is quite likely that subunit β is the polymerizing part of the enzyme. While γ and δ are also essential for the functioning of the enzyme [88], there is presently no evidence as to their role.

One of the most remarkable features of the viral RNA polymerase is its template specificity. Qβ replicase, which has been studied most intensively in this respect, will accept Qβ RNA and Qβ minus strands as template for replication, but not RNA from any other phage or virus [74,91]. Similar specificities have been described for other phage RNA replicases [87,92,93]. One might expect that the replicase would recognize its template by its 3′ terminal sequence, which is where initiation of the complementary strand takes place. Although certain simple requirements must be fulfilled at the 3′ end – it seems that a sequence of at least 3 CMP residues is necessary [94] –, these are not sufficient to ensure initiation on naturally occurring RNAs. In the case of Qβ RNA a region located at about the middle of the molecule is required for recognition by the replicase [95,96]. This region (which is different from the binding site involved in repression of protein synthesis by replicase) binds strongly to replicase, while the 3′ terminus itself has a comparatively very low affinity for the enzyme (H. Weber and C. Weissmann, unpublished results). Our model (fig. 5) for template recognition by replicase postulates that the strong interaction between the internal site on the RNA and the enzyme positions the 3′ end at the initiation site where chain initiation occurs. This hypothesis implies that template recognition need not depend on a very specific interaction between the enzyme and precisely defined nucleotide sequences but is mainly based on the relative positions of the internal binding site and the 3′ terminus of the RNA, i.e., on its tertiary structure. This model accounts for the fact that other RNAs, such as MS2 or f2 RNA which bind quite strongly to Qβ replicase [60,97,98] and have C-rich 3′ termini similar to that of Qβ RNA, nevertheless do not allow any initiation of complementary strand synthesis to occur.

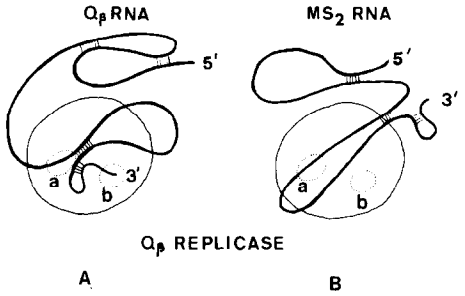

Fig. 5. Model for template recognition by Qβ replicase. Two sites on the enzyme (a and b) are required for template recognition (the site responsible for translational repression, cf. fig. 3, is not shown). (A) Productive interaction with Qβ RNA. Site (a) binds an internal region of the RNA tightly, placing the 3′ terminus in site (b), where it is weakly bound and chain initiation can taken place. (B) Non-productive interaction with heterologous RNA. Site (a) binds an internal region of the RNA for which it has some affinity, however the 3′ terminus is located far from site (b) and initiation cannot take place. The model is based on refs. [95] and [96].

It is not within the scope of this article to dwell on the many other remarkable features of phage RNA replication, however one more aspect of particular interest should be mentioned. The in vitro system of Qβ RNA replication is of sufficient fidelity and efficiency to allow the synthesis of infectious progeny RNA in large net excess over the input template [99]. This allows the virtually unlimited propagation of phage RNA in vitro and has opened the way for in vitro studies on evolution [100]. Moreover, tech-

niques have been developed to control the synthesis of RNA in such a way that nucleotide substitution can be generated in predetermined positions of the phage genome (Flavell, Sabo, Bandle and Weissmann, unpublished results). This directed mutagenesis is being used to generate point mutations in the extracistronic regions of Qβ RNA and should allow the elucidation of the hitherto unknown functions of these regions.

RNA, protein, get together

As in the case of other relatively simple viruses such as TMV, assembly of the phage components appears to proceed spontaneously, without a requirement for additional enzymes and factors. This conclusion is based on the finding [15] that in vitro renaturation of a mixture of phage RNA with denatured coat protein and A_2 (and in addition A_1 in the case of Qβ (Hofstetter, Monstein and Weissmann, unpublished results)) leads to the formation of infectious particles. The fact that in vitro reconstitution is very inefficient may be due to the denaturation and renaturation steps to which the components are subjected during their purification, but it is also possible that in vivo assembly proceeds more efficiently because RNA synthesis and packaging by viral proteins proceed coordinately.

Exit the phage

Forty to sixty minutes after infection the host cell is filled to bursting with virus particles, partially assembled phage components and virus-specific side products. The normally elongated cells are rounded off, indicating damage to the cell wall; finally lysis occurs, with the release of 10 000–40 000 particles, of which however only 10–50% are infectious. Cell lysis appears to depend on some unknown function of the phages' coat protein [101]. Curiously enough, infected bacteria kept at low temperature, 20–30°C rather than 37°C, produce phage at a very low rate. Virus is released into the medium through the undamaged cell wall; the infected cells do not lyse and continue to divide indefinitely [102].

Why all the fuss about RNA phages?

RNA phages are composed of only a few components, the detailed structure of which can be elucidated within reasonable time. This should make it possible to understand basic biological processes, such as translation and replication, as well as their regulation, on a truly molecular level. While some of the synthetic mechanisms and regulatory devices may prove to be unique to the particular system in which they were discovered, it is quite likely that much of the information gained and the technology developed will be applicable to the more complex eukaryotic systems, a field which needs all the help it can get.

Acknowledgement

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